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RESEARCH PAPER

BZR1 and BES1 participate in regulation of glucosinolate biosynthesis by brassinosteroids in *Arabidopsis*

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Abstract

The effect of 24-epibrassinolide (EBR) on glucosinolate biosynthesis in *Arabidopsis thaliana* was investigated in the present study by using mutants and transgenic plants involved in brassinosteroid (BR) biosynthesis and signal transduction, as well as glucosinolate biosynthesis. The results showed that EBR significantly decreased the contents of major aliphatic glucosinolates including glucoiberin (S3), glucoraphanin (S4), and glucoerucin (T4), as well as the indolic glucosinolates glucobrassicin (IM) and neoglucobrassicin (1IM). In addition, a significantly higher level of glucosinolates accumulated in the BR-deficient mutant *cpd* and a dramatically lower glucosinolate content in the transgenic plant *DWF4-ox* overexpressing the BR biosynthetic gene *DWF4* compared with their related wild-types, confirmed the repressing effect of BR on glucosinolate biosynthesis. BRI1, the receptor of BR signal transduction, was involved in regulation of glucosinolate biosynthesis by BR. Furthermore, the observation of reduced content of glucosinolates and lower expression levels of glucosinolate biosynthetic genes in *35S-BZR1/bzr1-1D* and *bes1-D* plants compared with the corresponding wild-types suggested that BZR1 and BES1, two important components in BR signal transduction, are responsible for the inhibiting role of BR in glucosinolate biosynthesis. The disappearance of the repressing effect of BR on glucosinolate content in the *myb28*, *myb34*, and *myb122* mutants indicated that these three MYB factors are important for the regulation of BR in glucosinolate biosynthesis.

Key words: *Arabidopsis thaliana*, brassinosteroids, BZR1, BES1, glucosinolates, MYB.

Introduction

Glucosinolates, a group of nitrogen and sulfur-containing secondary metabolites found mainly in Brassicaceae crops, has been proved to be involved in plant interaction with herbivores (Kliebenstein *et al*., 2002; Mewis *et al*., 2005) and micro-organisms (Kliebenstein *et al*., 2005; Brader *et al*., 2006). The main pathway and genes involved in glucosinolate biosynthesis have been identified using genetic tools and biochemical approaches in *Arabidopsis*. In the biosynthesis of the glucosinolate core structure, the substrates tryptophan and methionine are first metabolized by CYP79B2 and CYP79B3, or CYP79F1 and CYP79F2 (Du *et al*., 1995; Du and Halkier, 1996; Halkier and Du, 1997; Chen *et al*., 2003) to the corresponding aldoximes, which are further converted by the cytochrome P450 enzymes CYP83B1 and CYP83A1 to the next intermediate in indolic or aliphatic glucosinolate pathway (Bak and Feyereisen, 2001). Six MYB factors have been found to be transcriptional regulators in the biosynthesis of glucosinolate in *Arabidopsis*. Among these, MYB28,

Abbreviations: 1IM, neoglucobrassicin; 4IM: 4-methoxy glucobrassicin; BAK1, BRI1 associated receptor kinase 1; BES1, BRI1-EMS-suppressor 1; BKI1, BRI1 kinase inhibitor 1; BRI1, brassinosteroid-insensitive 1; BRs, brassinosteroids; BSKs, BR signalling kinases; BSU1, BRI1 and suppressor 1; BZR1, brassinazole-resistant 1; EBR, 24-epibrassinolide; IM, glucobrassicin; PP2A, protein phosphatase 2A; RT-PCR, real-time PCR; S3, glucoiberin; S4, glucoraphanin; S5, glucoalysin; S8, glucohirsutin.; SE, standard error; T4, glucoerucin.

MYB29, and MYB76 were shown to specifically *trans*-activate genes related to aliphatic glucosinolate biosynthetic pathway, i.e. *MAM3*, *CYP79F1*, and *CYP83A1* (Gigolashvili *et al*., 2007*b*, 2008). On the other hand, MYB34, MYB51, and MYB122, identified as regulators of the indolic glucosinolate biosynthetic pathway, exclusively *trans*-activate the promoters of *TSB1*, *CYP79B2*, and *CYP79B3*, which are involved in the indolic glucosinolate biosynthetic pathway (Celenza *et al*., 2005; Gigolashvili *et al*., 2007*a*).

The regulation of glucosinolate biosynthesis by plant hormones such as jasmonates and salicylic acid has been widely investigated (Kiddle *et al*., 1994; Doughty *et al*., 1995; Brader *et al*., 2001; Mikkelsen *et al*., 2003). Brassinosteroids (BRs), as stress alleviators distributed ubiquitously in the plant kingdom, protect plants from a variety of environmental stresses, including high- and low-temperature stress, drought, salinity, herbicidal injury, and insect and pathogen attack (Khripach *et al*., 2000; Bajguz and Hayat, 2009; Campos *et al*., 2009; Yang *et al*., 2011*b*). Although the physiological functions of BR in plants and their potential application in crops have been extensively studied, the role of BR in regulating glucosinolate and other secondary metabolites has not been reported. Great progress has been made in BR signal transduction in recent years by molecular genetic and proteomic surveys in *Arabidopsis*. The BR signal transduction pathway from the cell-surface receptor to nuclear gene expression has been elucidated. BR is perceived by the receptor kinase brassinosteroid insensitive 1 (BRI1) (Nam and Li, 2002). In the presence of BRs, the extracellular domain of BRI1 perceives BRs, leading to the dissociation of BRI1 kinase inhibitor 1 (BKI1) from plasma membrane, the association of BRI1-associated receptor kinase 1 (BAK1) with BRI1, and their *trans*-phosphorylation to form a completely active BR receptor complex (Yang *et al*., 2011*a*). The activation of BRI1 leads to sequential phosphorylation and activation of the BR-signalling kinases (BSKs) and members of the BRI1 and suppressor 1 (BSU1) family phosphatases (Kim and Wang, 2010; Kim *et al*., 2011), which inactivates the glycogen synthase kinase-3 enzymes through tyrosine dephosphorylation (Kim *et al*., 2009), allowing the activation of brassinazole-resistant 1 (BZR1) and BRI1-EMS-suppressor 1 (BES1) by protein phosphatase 2A (PP2A)-mediated dephosphorylation. Dephosphorylated BZR1 and BES1 are translocated into the nucleus to regulate over 1000 target genes (Sun *et al*., 2010). BZR1 and BES1 are essential in BR-regulated gene expression and physiological responses. Studies on BR signal transduction and its responsive genes have provided the clues for the regulation of BR in glucosinolate biosynthesis. Microarray chip data for the BR-responsive genes have shown that *CYP79B2*, an important gene involved in the biosynthesis of indolic glucosinolates (Chen and Andreasson, 2001), is downregulated by BR treatment in *Arabidopsis* (Goda *et al*., 2002). Moreover, analysis of the target genes of BZR1 has indicated that *MYB34* and *MYB51*, encoding transcriptional factors of indolic glucosinolate biosynthesis, both contain a BZR1-binding site in their promoters (Sun *et al*., 2010). In the present study, the role of BR in glucosinolate biosynthesis was investigated in the model plant *Arabidopsis*. Mutants and transgenic plants involved in BR biosynthesis and signal transduction as well as glucosinolate biosynthesis were selected to investigate how the biosynthesis of glucosinolate in *Arabidopsis* is modulated by BR, and a possible genetic pathway involved in the regulation of glucosinolate biosynthesis by BR was also elucidated.

Materials and methods

Plants and growth conditions

Seeds were sterilized for 30 s in 75% ethanol and washed with sterile H_2O until neutral, and then immersed in 10% sodium hypochlorite for 3 min, followed by washing with sterile H_2O five times. The seeds were stratified for 3 d at 4 °C and transferred into flasks with liquid growth medium (1/2 sterilized Murashige–Skoog salt solution+1% sucrose), or transferred into Petri dishes with growth medium (1/2 sterilized Murashige–Skoog salt solution+1% sucrose+0.7% agar). Plants were grown with a photoperiod of 16 h light/8 h dark (110 μm photons m^{-2}) in a plant growth chamber at 23 °C. After 10 d, the flask seedlings were drenched with or without 24-epibrassinolide (EBR; Sigma-Aldrich, St Louis, USA) at different concentrations for 3 d. The control was treated with 0.07% ethanol, which is a solvent of EBR. The Petri dish seedlings were grown for 13 d and then collected to analyse the glucosinolate content and the expression level of related genes. Using two methods of planting *Arabidopsis thaliana* was due to the special phenotypes of BR-related mutants and transgenic plants such as *bes1-D*, *DWF4-ox*, and *35S-BZR1/bzr1-1D*, which are too big for liquid cultivation, as well as *cpd* and *bri1* which are too small for liquid cultivation.

Seeds of *bri1-5*, *DWF4-ox*, *bes1-D*, and *35S-BZR1/bzr1-1D* were obtained from Dr Zhiyong Wang (Department of Plant Biology, Carnegie Institution for Science, Stanford University, CA, USA), and *cpd* (SALK_090732C), *myb28* (SALK_136312), *myb29* (CS121027), *myb34* (SALK_006901), *myb51* (SALK_045103), and *myb122* (SALK_022993) were provided by the Arabidopsis Biological Resource Center. The genetic background of all mutants was Columbia (Col-0) but harvested under different conditions, except for *bri1-5*, whose genetic background was Wassilewskija (Ws).

Glucosinolate assay

Glucosinolates were extracted and analysed as described previously with minor modifications (Guo *et al*., 2011; Miao *et al*., 2013). Samples (200 mg) were boiled in 1 ml of H_2O for 10 min. After transferring the supernatant to a new tube, the residues were washed with 1 ml of H_2O and the combined aqueous extract was applied to a DEAE–Sephadex A-25 (35 mg) column (pyridine acetate form) (Sigma-Aldrich). Sinigrin (Sigma-Aldrich) was used as an internal standard for HPLC analysis. Desulfoglucosinolates were identified by comparison of retention time and quantified by peak area. The glucosinolate concentration was expressed as μmol g^{-1} of fresh weight of *Arabidopsis* seedlings.

RNA extraction

Total RNA was isolated from *Arabidopsis* seedlings six times for two biological repeats using RNAiso Plus (Takara, Japan) according to the manufacturer's instruction. A total of 1 ml of RNAiso Plus was added to the cell pellet. After resuspending the cells, the tubes were shaken vigorously at room temperature for 5 min. After centrifugation at 12 000*g* at 4 °C for 5 min, the samples were immediately added to 200 μl of chloroform, shaken, and stored at room temperature for 5 min. The extraction mix was then centrifuged for 15 min at 12 000*g* at 4 °C to promote phase separation. The aqueous phase was retrieved and mixed with an equal volume of isopropanol, incubated at room temperature, and centrifuged to concentrate the precipitated RNA. The RNA pellet was washed using 75% ethanol, air dried, and dissolved in diethylpyrocarbonate H_2O.

Real-time PCR (RT-PCR) analysis

RNA samples were reverse transcribed into cDNA. The synthesized cDNAs were diluted ten times in H_2O and their concentrations were normalized based on the amplification of *AtActin*. RT-PCR was performed with a total volume of 25 µl, which contained 1 µl of diluted cDNA, 1µl of each 5 µM forward and reverse primer, 9.5 µl of ddH_2O and 12.5µl of SYBR Green PCR Master Mix (Takara) on an ABI PRISM Step One Plus TM Real-Time PCR System. PCR amplification was performed using cycling conditions of 95 °C for 30 s, followed by 40 cycles of 95 °C for 5 s and 58 °C for 1 min. The expression level of *Arabidopsis ACTIN2* was used as an internal control and the expression of other genes was computed with the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001). The primers used are listed in Table 1.

Statistical analysis

Statistical analysis was performed using the SPSS package program version 11.5 (SPSS, Chicago, IL, USA). For Fig. 1, the data were analysed by one-way analysis of variance, followed by Turkey's HSD multiple comparison test. For Figs 2–5 and 7, the data were analysed using an independent-samples *t*-test. The values are reported as means ±standard error (SE) for all results. Differences were considered significant at $P < 0.05$.

Results

Effect of EBR on glucosinolate content in Arabidopsis

To study the role of BR on glucosinolate biosynthesis, 10-d-old Col-0 seedlings were treated with EBR at different concentrations (1, 3, and 5 µM) for 3 d. As shown in Fig. 1, the influence of EBR on glucosinolate accumulation was concentration dependent, and treatment with 3 µM EBR was the most effective in inhibiting the biosynthesis of glucosinolate. The contents of the aliphatic glucosinolates glucoiberin (S3), glucoraphanin (S4), and glucoerucin (T4), as well as the indolic glucosinolates glucobrassicin (IM) and neoglucobrassicin (1IM), all decreased significantly after treatment with 3 µM EBR.

Table 1. Primers used for RT-PCR. F, Forward; R, reverse.

Gene name	Locus code	Sequence (5'→3')
MYB34	At5g60890	F: CGGGTCTTAAGTAATTAGCC
		R: AAGAAAGGAGCTTGGACTCC
MYB51	At1g18570	F: ACAAATGGTCTGCTATAGCT
		R: CTTGTGTGTAACTGGATCAA
MYB122	At1g74080	F: TCCGTTGAGTCTTGTTTGGA
		R: TTGTCAATCCCTTCACAGGA
MYB28	At5g61420	F: TCCCTGACAAATACTCTTGCTGAT
		R: CATTGTGGTTATCTCCTCCGAATT
MYB29	At5g07690	F: CAATACTGGAGGAGGATATAACC
		R: AGTTCTTGTCGTCATAATCTTGG
MYB76	At5g07700	F: ACGTTTAATCGATGATGGCA
		R: ATGGGCTCAACTGGATTAGG
CYP79F1	At1g16410	F: CCATACCCTTTTCACATCCTACTGTCT
		R: GTAGATTGCCGAGGATGGGC
CYP79F2	At1g16400	F: ACTAGGATTTATCGTCTTCATCGCA
		R: CTAGGACGAGTCATGATTAGTTCGG
CYP79B2	At4g39950	F: TTTGATGGATTGTCTGGCGC
		R: CAAAGACGAACAAGGCAACC
CYP79B3	At2g22330	F: CGGTTTGTTTATCATCTCCGC
		R: TTGCTTACCGCTGATGAAATC
CYP83A1	At4g13770	F: TTCAAGAGGTTGTCAATGAGACGC
		R: CTACAATATCCAAGATGACGGCTTT
CYP83B1	At4g31500	F: TCCGACCTTTTCCCTTATTTCG
		R: TTGAGACGTGCACTGAGACCAG
ACTIN2	At3g18780	F: TAACTCTCCCGCTATGTATGTCGC
		R: CCACTGAGCACAATGTTACCGTAC

Glucosinolate accumulation in cpd and DWF4-ox

To investigate further the inhibiting role of BR in glucosinolate accumulation, plants containing different levels of BR were used in our experiment, and the results showed that the contents of aliphatic and indolic glucosinolates increased in the BR-deficient mutant *cpd* and decreased in the BR-overproducing plant *DWF4-ox* (Wang *et al*., 2002) compared with their corresponding wild-type (Figs 2 and 3), indicating that the endogenous BR level affected the accumulation of glucosinolate.

BRI1 is involved in the regulation of glucosinolate by EBR

BRI1, a kinase with an extracellular leucine-rich repeat and a transmembrane domain, has been shown to be the receptor of the BR-signalling pathway (Nam and Li, 2002). The BRI1 mutant *bri1-5* (a weak allele) was used to identify whether the regulation of glucosinolate accumulation by BR was via the BRI-signalling pathway. As shown in Fig. 4, the S3 content, a predominant glucosinolate in Ws, as well as that of the indolic glucosinolates IM and 1IM in the *bri1-5* mutant were significantly increased compared with that of the wild-type Ws.

Glucosinolate accumulation in BR-signalling transduction mutants

The nuclear protein BZR1 and its homologue BES1, two important transcriptional factors in the BR-signalling pathway, regulating BR-response genes by binding to their DNA promoters (Wang *et al*., 2002). The dominant *Arabidopsis* mutant *brassinazole-resistant 1-1D* (*bzr1-1D*) stabilizes the nuclear protein encoded by *BZR1*, and transformation of the mutant *BZR1* gene into Col-0 endows *35S-BZR1/bzr1-1D* with a gain-of-function nature in BR-regulated responses. In addition, the BES1 protein is stabilized and accumulates at high levels in the mutant *bri1-EMS-suppressor 1-D* (*bes1-D*). In the current study, the accumulation of both aliphatic and indolic glucosinolates was decreased significantly in *35S-BZR1/bzr1-1D* and *bes1-D* compared with the wild type (Fig. 5), indicating the key roles of BZR1 and BES1 in regulating the biosynthesis of glucosinolate in *Arabidopsis*.

Expression of glucosinolate-related genes in 35S-BZR1/bzr1-1D and bes1-D

The expression pattern of transcriptional factors and biosynthetic genes in the glucosinolate biosynthesis pathway was measured by RT-PCR in *35S-BZR1/bzr1-1D* (Wang *et al*., 2002) and *bes1-D* (Yin *et al*., 2002) plants. Six MYB transcription

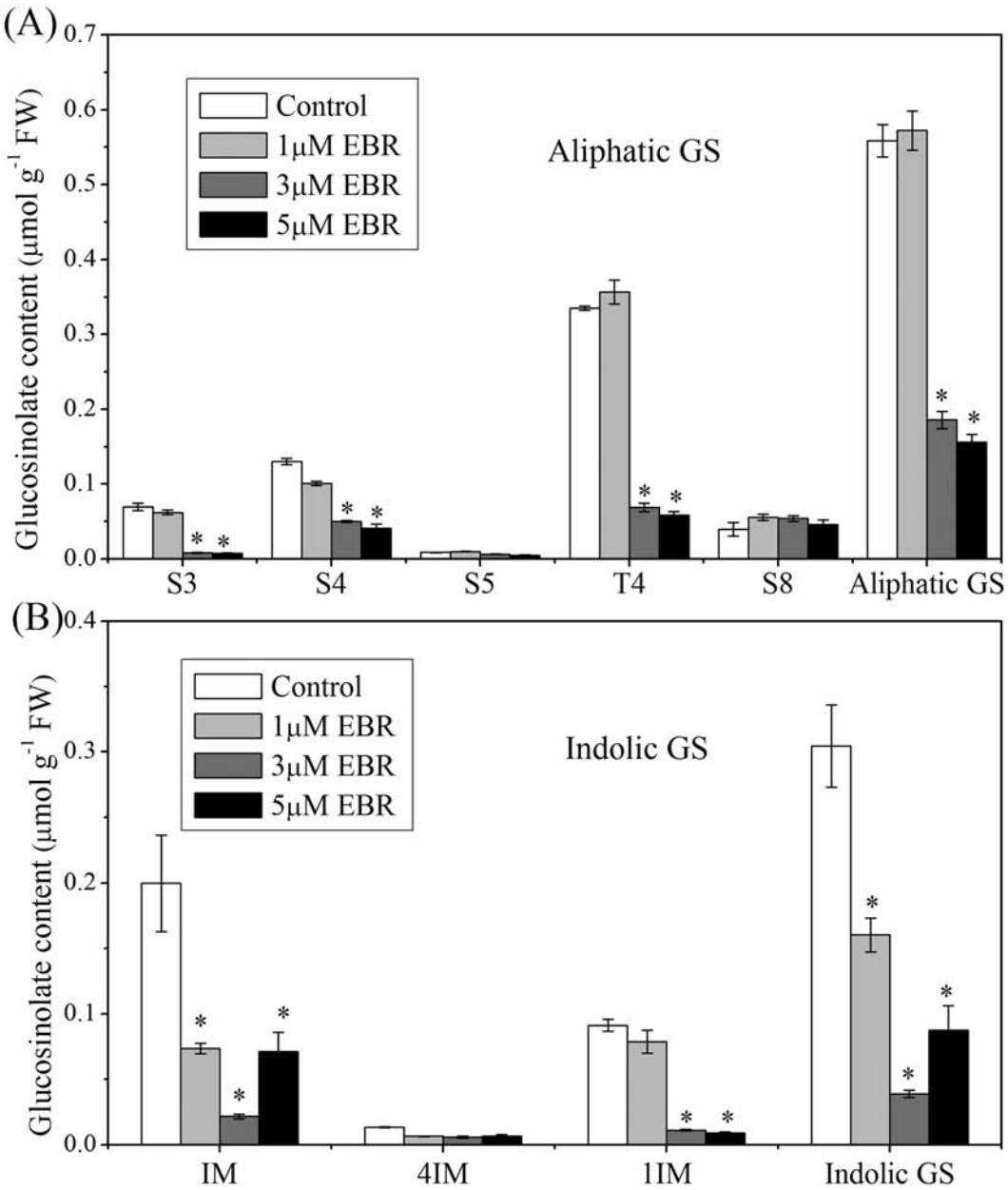

Fig. 1. Effect of EBR treatment at different concentrations on glucosinolate (GS) contents. (A) Individual and total aliphatic GS content were measured in 13-d-old *Arabidopsis* seedlings treated with 1, 3, and 5 μM EBR for 3 d. (B) Individual and total indolic GS content were measured in 13-d-old *Arabidopsis* seedlings treated with 1, 3, and 5 μM EBR for 3 d. Each result is the mean±SE of four replicates per treatment. Values marked with an asterisk wre significantly different from the control (P <0.05). S3, glucoiberin; S4, glucoraphanin; S5, glucoalysin; T4, glucoerucin; S8, glucohirsutin; IM, glucobrassicin; 4IM, 4-methoxy glucobrassicin; 1IM, neoglucobrassicin.

factors have been demonstrated to regulate the biosynthesis of glucosinolate in *Arabidopsis* (Yan and Chen, 2007). Among these, MYB28, MYB29, and MYB76 are related to the regulation of aliphatic glucosinolate biosynthesis, whilst MYB34, MYB51, and MYB122 are involved in indolic glucosinolate biosynthesis. As shown in Fig. 6A, the expression levels of the six *MYB* genes were all significantly decreased in *35S-BZR1/bzr1-1D* and *bes1-D*, except for *MYB28* in *bes1-D*.

Gene expression of the main biosynthetic genes such as *CYP79B2*, *CYP79B3*, *CYP79F1*, *CYP79F2*, *CYP83A1*, and *CYP83B1* was also detected in the present study (Fig. 6B), and the results showed that all the biosynthetic genes tested were downregulated in *35S-BZR1/bzr1-1D* and *bes1-D* compared with the wild type, which is in accordance with the reduction of glucosinolate content in *35S-BZR1/bzr1-1D* and *bes1-D*.

Effect of BR on glucosinolate accumulation in myb28, myb29, myb34, myb51, and myb122 mutants

To investigate whether the transcriptional factors involved in glucosinolate biosynthetic pathway are essential to the regulation of BR in glucosinolate accumulation in *Arabidopsis*,

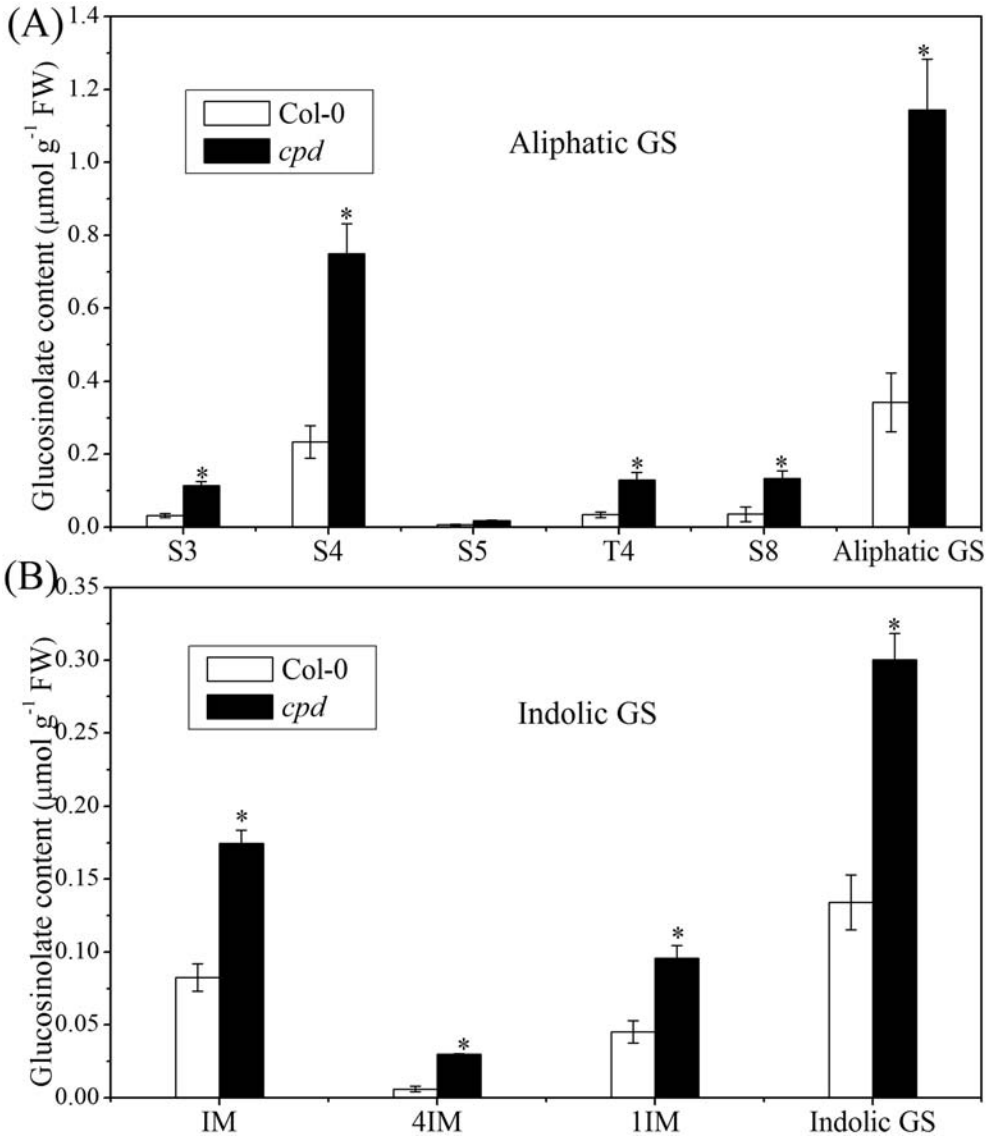

Fig. 2. Composition and content of glucosinolate (GS) in the BR-deficient mutant *cpd*. (A) The aliphatic GS profile was analysed in 18-d-old *cpd* and its corresponding wild-type. (B) The indolic GS profile was analysed in 18-d-old *cpd* and its corresponding wild type. Each result is the mean±SE of four replicates per treatment. Values marked with an asterisk are significantly different from the control (P <0.05).

changes in the content of glucosinolates after treatment with EBR were analysed in five mutants *myb28*, *myb29*, *myb34*, *myb51*, and *myb122*. As shown in Fig. 7A, the level of total aliphatic glucosinolates decreased dramatically in *myb28* and *myb29*, especially in *myb28*. The repressing effect of BR on aliphatic glucosinolates accumulation was diminished in *myb28*. The indolic glucosinolate content was reduced significantly in *myb34*, *myb51*, and *myb122*, and the effect of BR on indolic glucosinolates was weakened in *myb34* and *myb122* (Fig. 7B).

Discussion

EBR decreases the accumulation of glucosinolates in Arabidopsis

BRs are known to alleviate plants from stress by transcriptional regulation of plant development and resistance (Khripach *et al.*, 2000; Bajguz and Hayat, 2009; Campos *et al.*, 2009; Yang *et al.*, 2011*b*). Secondary metabolites also function in defence against difference kinds of abiotic or biotic stress (Kliebenstein, 2004). However, little has been reported about whether BR is involved in the regulation of secondary metabolites biosynthesis. Here, we took glucosinolates as an example to elucidate how BR regulates the biosynthesis of secondary metabolites. In the present study, we found that BR reduced the contents of both aliphatic and indolic glucosinolates. The accumulation of S4 and T4 (Fig. 1A), major aliphatic glucosinolates, as well as the indolic glucosinolates IM and 1IM (Fig. 1B), in *Arabidopsis* was significantly decreased after treatment with BR. Furthermore, the significantly higher glucosinolate content in the BR-deficient mutant *cpd* (Fig. 2) and dramatically lower glucosinolate content in the transgenic plant *DWF4-ox* overexpressing *DWF4* (Fig. 3) confirmed the inhibiting role of BR in glucosinolate biosynthesis. This is in

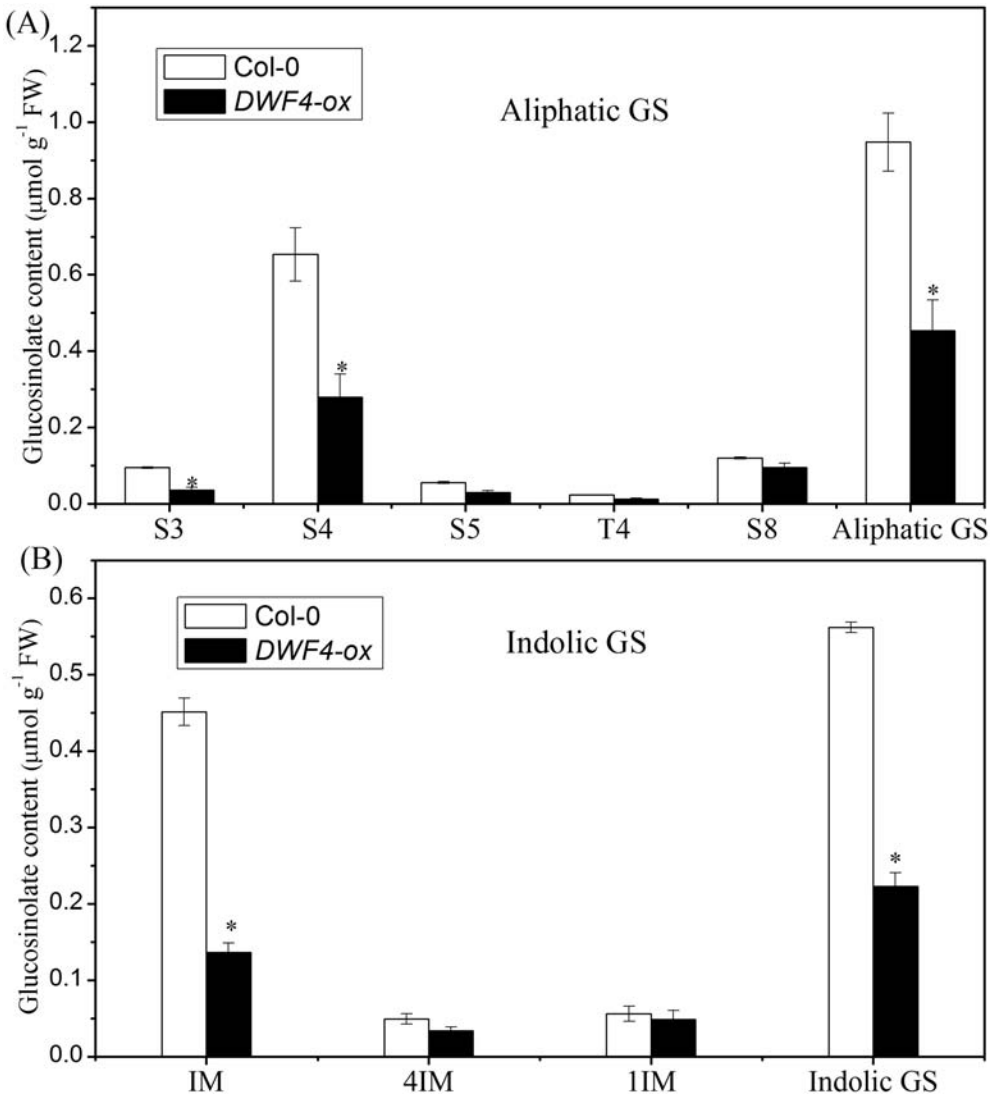

Fig. 3. Composition and content of glucosinolate (GS) in the BR-overproducing plant *DWF4-ox*. (A) The aliphatic GS profile was analysed in 13-d-old *DWF4-ox* and its corresponding wild type. (B) The indolic GS profile was analysed in 13-d-old *DWF4-ox* and its corresponding wild type. Each result is the mean±SE of four replicates per treatment. Values marked with an asterisk are significantly different from the control (P <0.05).

accordance with the results of microarray chip data by Goda *et al.* (2002), which indicated that *CYP79B2*, an important gene in indolic glucosinolate biosynthesis, was downregulated by the application of BR. Thus, we hypothesized that BR inhibits glucosinolate biosynthesis in *Arabidopsis*.

BRI1-dependent regulation of glucosinolate biosynthesis by EBR

BRI1 with an extracellular domain connected by a single-pass transmembrane helix to an intracellular serine/threonine kinase domain encoding a leucine-rich repeat receptor-like kinase is essential for BR perception at the cell surface (Nam and Li, 2002). In the BR-perception mutant *bri1-5* (a weak *bri1* allele), the signal for BR is cut off and the mutant exhibits the phenotype of dwarfism and dark-green leaves. In our study, the absence of BRI1 increased the content of aliphatic and indolic glucosinolates (Fig. 4), indicating that BRI1 participates in the regulation of glucosinolates by BR. In addition, Sun *et al.* (2010) found that 80% of the genes affected in *bri1-116* were affected in opposite ways by *bzr1-1D*, indicating that BZR1 directly or indirectly regulates the expression of about 80% of the genes controlled by BR through BRI1.

BZR1 and BES1 are involved in the regulation of glucosinolate biosynthesis by EBR

Genetic studies have revealed a central role for BZR1 and BES1 in BR-regulated plant growth (Wang *et al.*, 2002; Yin *et al.*, 2002). Activation of either BZR1 or BES1 by dominant mutations (*bzr1-1D* and *bes1-D*) suppresses nearly all phenotypes of the BR-insensitive *bri1* mutant (Wang *et al.*, 2002; Yin *et al.*, 2002).

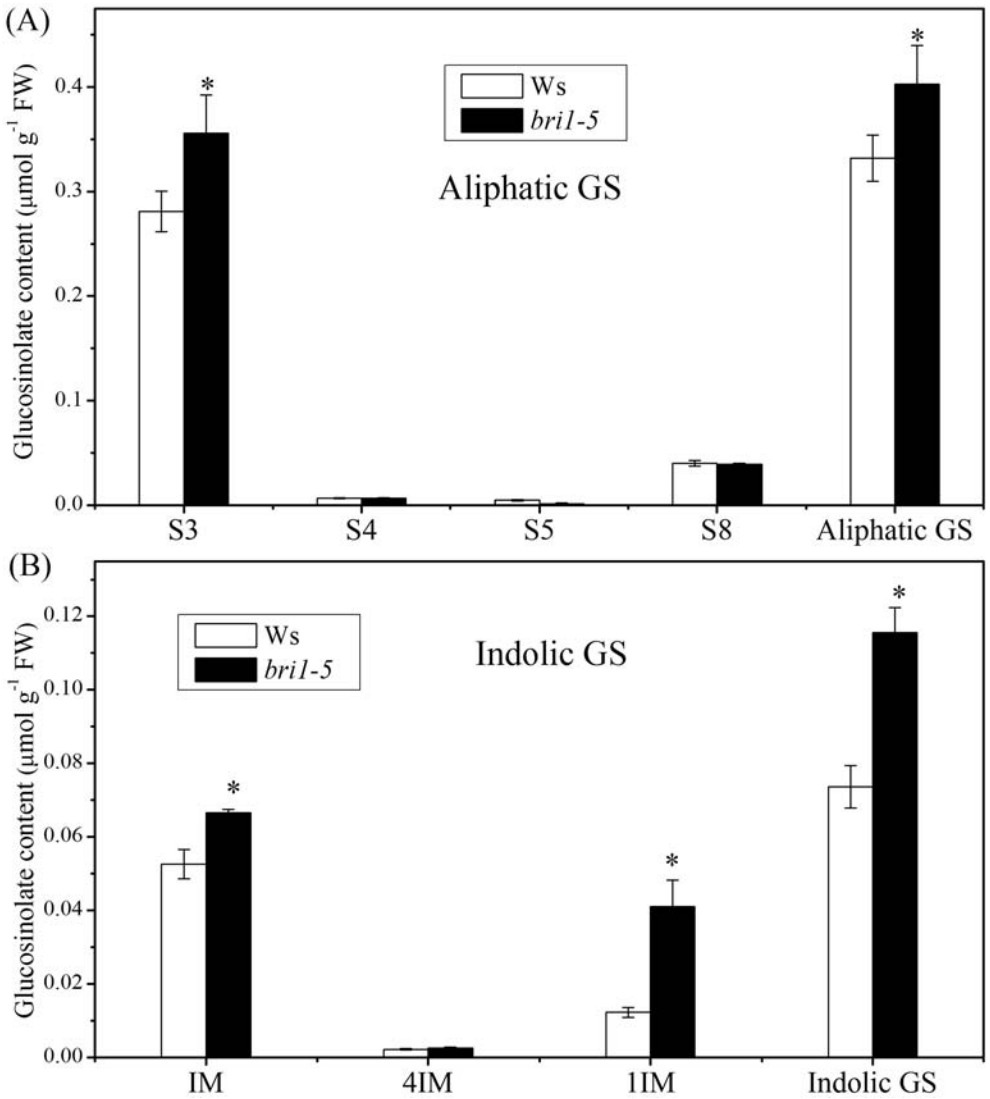

Fig. 4. Composition and content of glucosinolate (GS) in the BR receptor mutant *bri1-5*. (A) The aliphatic GS profile was analysed in 13-d-old *bri1-5* and its corresponding wild type. (B) The indolic GS profile was analysed in 13-d-old *bri1-5* and its corresponding wild type. Each result is the mean±SE of four replicates per treatment. Values marked with an asterisk are significantly different from the control (P <0.05).

Glucosinolate accumulation in *35S-BZR1/bzr1-1D* and *bes1-D* was analysed in our study to elucidate whether these two important transcriptional factors are involved in the BR-regulated glucosinolate accumulation. The results showed that the total aliphatic and indolic glucosinolates were all significantly reduced in these two plants compared with those in the wild type, indicating that BZR1 and BES1 play key roles in BR-regulated glucosinolate biosynthesis. This was further confirmed by analysis of target genes of BZR1 by microarray chip experiments (Sun *et al*., 2010), which showed that both *MYB34* and *MYB51*, two transcriptional factors in indolic glucosinolate biosynthesis, are BR-responsive genes.

BZR1 and BES1 share 88% sequence identity at the protein level and bind to DNA through the conserved N-terminal DNA-binding domain (He *et al*., 2005). They have very similar biochemical functions. ChIP-qPCR experiments have shown that both BZR1 and BES1 bind to the BR-repressed gene *DWF4* and the BR-induced gene *SAUR-AC1* (Sun *et al*., 2010), and BES1 binds to 18 of the 19 BZR1-binding sites analysed (Sun *et al*., 2010). The DNA-binding activity BZR1 and its nuclear localization are tightly controlled by BR signalling (Kim and Wang, 2010), and the genes regulated by BZR1 should be responsive to BR treatment or affected in BR mutants. The expression of glucosinolate biosynthetic genes in *35S-BZR1/bzr1-1D* and *bes1-D* was investigated in our study, and all the tested genes including six MYB transcriptional factors and six main biosynthetic genes were down-regulated in these two plants. These six MYB transcriptional factors, MYB28, MYB29, MYB76, MYB34, MYB51, and MYB122, might function in the nucleus due to an amino acid sequence acting as an SV40-type nuclear localization signal motif (Gigolashvili *et al*., 2007*b*). Among these, MYB28 and MYB34 are the main transcriptional regulators in the aliphatic and indolic glucosinolate biosynthesis pathways, respectively. The downstream genes of the glucosinolate biosynthetic pathway, *CYP79F1*, *CYP79F2*, and *CYP83B1*, as well as

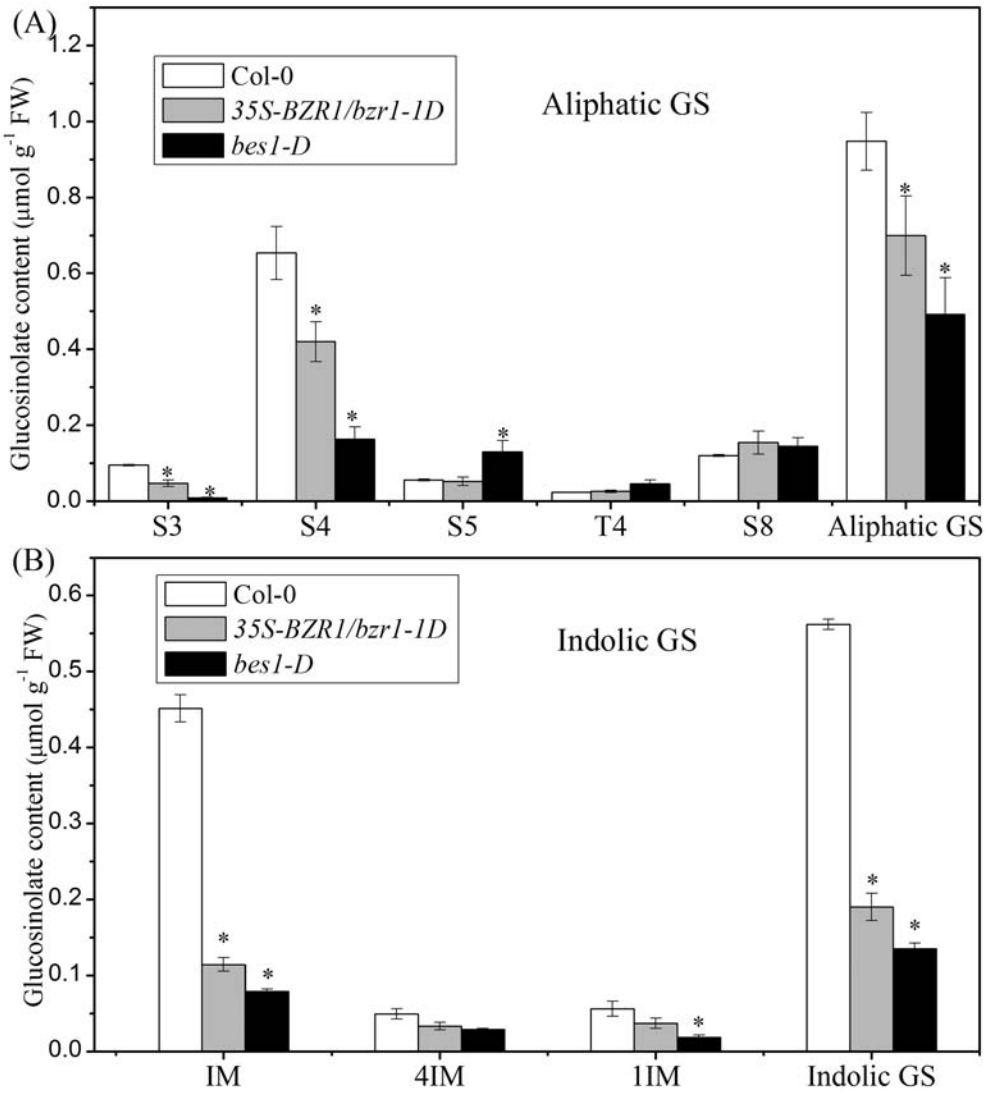

Fig. 5. Composition and content of glucosinolate (GS) in the BR signal transduction plants *35S-BZR1/bzr1-1D* and *bes1-D*. (A) The aliphatic GS profile was analysed in 13-d-old *35S-BZR1/bzr1-1D* and *bes1-D* and their corresponding wild type. (B) The indolic GS profile was analysed in 13-d-old *35S-BZR1/bzr1-1D* and *bes1-D* and their corresponding wild type. Each result is the mean±SE of four replicates per treatment. Values marked with an asterisk are significantly different from the control (P <0.05).

CYP79B2 and *CYP79B3*, are positively mediated by MYB28 and MYB34. Thus, we propose that BZR1 and BES1 might mediate the regulation of the glucosinolate biosynthetic pathway at the transcriptional level via the transcriptional factors or by binding directly to the biosynthetic genes (Fig. 8), such as *CYP79B2*, which has been shown to be responsive to BZR1 in microarray chip analysis (Sun *et al.*, 2010). Therefore, the inhibiting effect of BR on glucosinolate accumulation could be due to the transcription repressor function of BZR1 and BES1 on glucosinolate biosynthetic genes.

Downregulation of glucosinolates by EBR through MYB transcription factors

To elucidate the role of the MYB transcriptional factors in BR-inhibited glucosinolate accumulation, five MYB mutants, *myb28*, *myb29*, *myb34*, *myb51*, and *myb122* were used to elucidate the effect of BR on their glucosinolate accumulation. As shown in Fig. 7B, MYB34, MYB51 and MYB122 loss-of-function mutation all conferred reduced indolic glucosinolate levels. The deficiency of MYB34 and MYB122 lost the responsive ability to the application of BR, and there was no detectable reduction in indolic glucosinolates in mutants *myb34* and *myb122* after treatment with BR, indicating an important role of MYB34 and MYB122 in the BZR1-mediated repression of glucosinolate accumulation. The expression of *MYB34* has the potential to be controlled at the translational as well as the transcriptional level, because the MYB34 transcript contains three short open reading frames upstream and out of frame with the Myb-encoding open reading frame (Bender and Fink, 1998). As for the aliphatic glucosinolates, the deficiency in MYB28 and MYB29 led to a great decrease in the content of aliphatic glucosinolate compared with that of the wild type (Fig. 7A). Both MYB28 and MYB29 are transcriptional

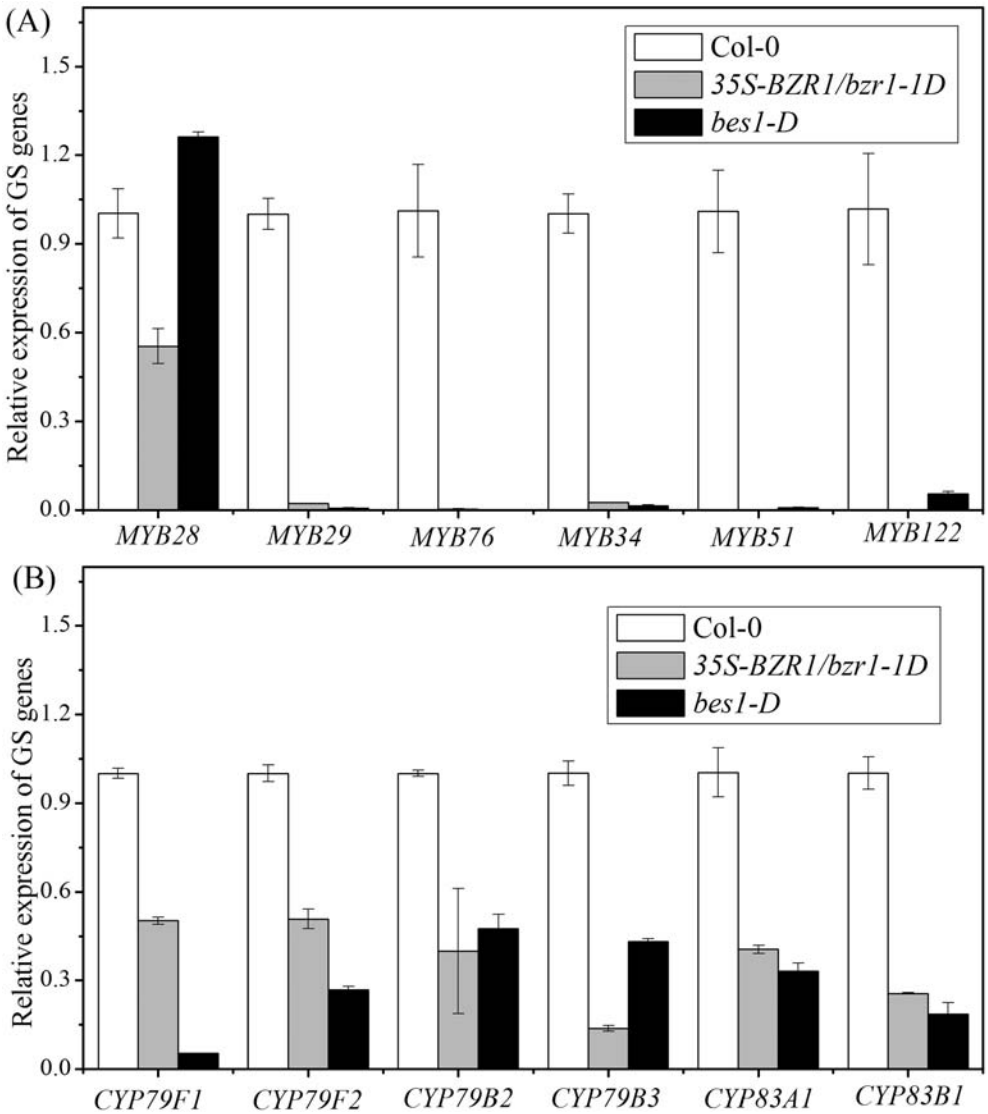

Fig. 6. The expression of genes involved in glucosinolate (GS) biosynthesis in BR signal transduction mutant plants *35S-BZR1/bzr1-1D* and *bes1-D* and their corresponding wild type. (A) The mRNA levels of *MYB28*, *MYB29*, and *MYB76* were measured in 13-d-old seedlings. (B) The mRNA levels of *CYP79B2*, *CYP79B3*, *CYP79F1*, *CYP79F2*, *CYP83A1*, and *CYP83B1* were measured in 13-d-old seedlings. Relative expression level was measured by RT-PCR. Results are shown as the mean transcript level from two biological replicates. In order to allow an easier comparison, we set the expression level of the control samples to '1'.

factors regulating the biosynthesis of aliphatic glucosinolates (Gigolashvili *et al*., 2007*b*). Hirai *et al*. (2007) found that MYB28 and MYB29 have functional redundancy in the regulation of aliphatic glucosinolate, in which MYB28 is a key component of this pathway whilst MYB29 is accessory. The repressing effect of BR on glucosinolates was diminished in mutant *myb28*, indicating that MYB28 plays a key role in the BR-regulated decrease in glucosinolates.

Conclusions

BR inhibited the biosynthesis of glucosinolates, and the endogenous content of BR affected the accumulation of glucosinolates in *A. thaliana*. Furthermore, the regulation of BR in glucosinolates biosynthesis was via BRI1, the membrane receptor. BZR1 and BES1, two important transcriptional factors downstream of BRI1 in the BR signal transduction pathway, were both involved in the regulation of glucosinolates by BR, as lower levels of glucosinolate were observed in *35S-BZR1/bzr1-1D* and *bes1-D* plants compared with those in the wild type. In addition, the downregulation of genes related to the glucosinolate biosynthetic pathway in *35S-BZR1/bzr1-1D* and *bes1-D* combined with the former microarray chip data (Chen and Andreasson, 2001) suggested that BZR1 and BES1 might regulate glucosinolate biosynthesis directly by binding to glucosinolate biosynthetic genes by the conserved N-terminal DNA-binding domain or indirectly through MYB factors. The disappearance of the repressing effect of BR on glucosinolate contents in *myb28*, *myb34*, and *myb122* mutant plants indicated that these three MYB factors are

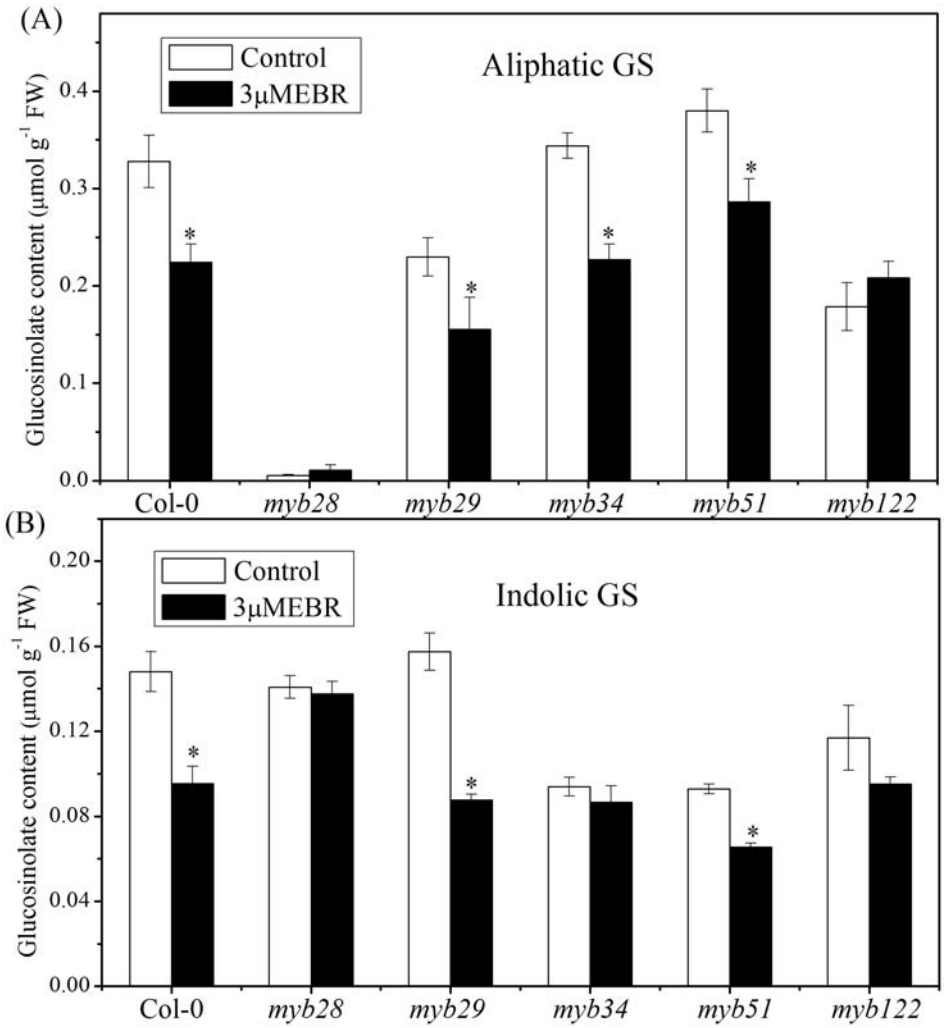

Fig. 7. Effect of EBR on glucosinolate (GS) contents in *Arabidopsis*. (A) The total aliphatic GS level was measured in 13-d-old seedlings of *Arabidopsis* glucosinolate mutants and the related wild type treated with 3 μM EBR for 3d. (B) The total indolic GS level was measured in 13-d-old seedlings of *Arabidopsis* glucosinolate mutants and the related wild type treated with 3 μM EBR for 3d. Each result is the mean±SE of four replicates per treatment. Values marked with an asterisk are significantly different from the control (P <0.05).

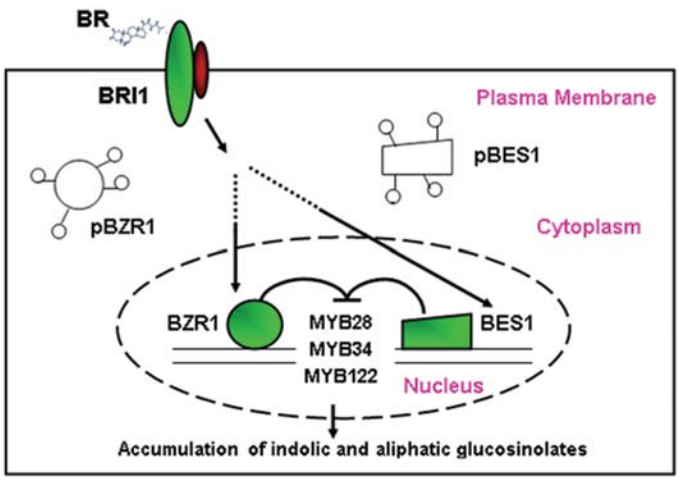

Fig. 8. The possible mechanism involved in regulation of glucosinolate biosynthesis by BR in *Arabidopsis*. (This Fig. is available in colour at *JXB* online.)

important for BR-regulated indolic glucosinolate accumulation. However, more work is needed to further elucidate how BZR1 and BES1 regulate the biosynthesis of glucosinolates at the molecular level in *Arabidopsis*.

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